Baculovirus Expression of *Bm*AChE3, a cDNA Encoding an Acetylcholinesterase of *Boophilus microplus* (Acari: Ixodidae)

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ABSTRACT The complete cDNA sequence encoding a *Boophilus microplus* (Canestrini) (Acari: Ixodidae) acetylcholinesterase (AChE3) was expressed in the baculovirus system. The recombinant AChE3 protein (r*Bm*AChE3) was secreted as a soluble form into the cell culture medium and was identified as a functional AChE by substrate specificity and by inhibition with the AChE-specific inhibitors eserine sulfate and BW284c51. Inhibition kinetics of r*Bm*AChE3, in the presence of the organophosphate paraoxon, revealed sensitivity comparable with that of adult, organophosphate-susceptible neural AChE. To our knowledge, this is the first report of the cloning and successful expression of a functional ixodid AChE.

KEY WORDS acetylcholinesterase, tick, Acari, Ixodidae, kinetics

The southern cattle tick, *Boophilus microplus* (Canestrini) (Acari: Ixodidae), is an ectoparasite of cattle that vectors the causative agent of bovine babesiosis, *Babesia bovis* and *Babesia bigemina* (Smith and Kilborne 1893), and was eradicated from the United States in 1943 (Graham and Hourrigan 1977). *B. microplus* remains endemic to Mexico, and intermittent outbreaks still occur in eight Texas counties adjacent to the U.S.–Mexico border in deep south Texas. However, permanent reestablishment of *B. microplus* in the United States has been prevented by a surveillance and quarantine program that is maintained by Veterinary Services branch of the Animal Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture. All cattle imported into the United States from Mexico are required to be dipped in vats containing the organophosphate (OP) coumaphos (George 1996). There is increasing concern over reports of OP resistance in Mexico (Santamaria and Fragoso 1994, Fragoso et al. 1995) and the potential failure of the United States entry barrier to *B. microplus* (Davey et al. 2003).

The physiological target site for OP toxicity is the quasi-irreversible inhibition of acetylcholinesterase (AChE; O'Brien 1967, p. 332). The first report of AChE insensitivity to OP inhibition in *B. microplus* was reported by Lee and Bantham (1966) and was considered the principal resistance mechanism in *B. microplus* (Bull and Ahrens 1988). In *Drosophila*, point mutations within the AChE gene result in amino acid substitutions that alter the conformation of AChE and thereby the rate at which it is inhibited by OP (Morton 1993, Fournier and Mutero 1994). Pruett (2002), evaluating the kinetic analysis of OP inhibition of AChE extracted from OP-resistant *B. microplus* strains, observed that the slower rate of OP inhibition of insensitive AChE was most affected by a slower rate of enzyme phosphorylation.

Three cDNAs that encode putative AChEs from *B. microplus* (*Bm*AChE1, *Bm*AChE2, and *Bm*AChE3) have been identified, but to date, no molecular basis for altered AChE activity has been defined, i.e., OP-insensitive AChE (Baxter and Barker 1998, Hernandez et al. 1999, Temeyer et al. 2004). Identification of the putative *Bm*AChEs was based, in silico, on conservation of amino acid residues, including the presence and spacing of amino acids comprising the catalytic triad and disulfide bonds, presence of a presumptive signal peptide and stretches of amino acid sequences homologous to known AChE conserved amino acid sequences, size of the predicted mature peptide, and other properties common to known AChEs. Many of these same properties are shared by non-AChE members of the AChE gene family (Oakeshott et al. 1999), reducing the certainty of the correct designation of the *Bm*AChE genes as encoding functional AChEs. In addition, none of the *Bm*AChE cDNAs have been verified with respect to expression and enzymatic properties.

The present work reports baculovirus expression of the *Bm*AChE3 cDNA and biochemical characterization of the acetylcholinesterase it encodes. Results of this study, for the first time, enable the direct linkage of genetic and biochemical data on acetylcholinesterase, which may potentially lead to elucidation of the

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mechanism of organophosphate resistance in this important ectoparasitic arthropod.

Materials and Methods

Tick Material. *B. microplus* ticks were maintained at the Cattle Fever Tick Research Laboratory (CFTRL, Moore Field, TX). This study used ticks from the Muñoz strain, collected from an outbreak in Zapata County, Texas, and established at the CFTRL in 1999. The Muñoz and Gonzalez strains (Zapata County outbreak, 1994) were both characterized as susceptible to all major classes of acaricides, and they have been used as reference strains for comparison with various acaricide-resistant strains (Li et al. 2003).

Cloning and Sequencing. Total RNA was isolated from *B. microplus* larvae that were ground to a powder by using a liquid nitrogen-cooled mortar and pestle. Total RNA was isolated from the larval powder using Tri Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. A gene-specific primer (*Bm*AChE2273L19, 5′-GCTATCATGAGCATGTTTC-3′), designed from the *B. microplus* AChE3 sequence (Temeyer et al. 2004; GenBank accession no. AY267337), was used to direct synthesis of first-strand cDNA from the larval RNA template by using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).

The complete coding region for *Bm*AChE3 was amplified from cDNA by two rounds of high-fidelity polymerase chain reaction (PCR) by using iPROOF DNA polymerase (Bio-Rad, Hercules, CA), according to the manufacturer's instructions, with primer pair *Bm*AChE61U15 (5′-CGGTGACCACAGTGC-3′) and *Bm*AChE32305L16 (5′-GCTATCATGAGCATGTTTC-3′), followed by nested PCR by using primers *Bm*AChE150U15 (5′-GGGGAGCACGGTA-3′) and *Bm*AChE2246L15 (5′-GCCGTAACAGTGGAA-3′). The amplified cDNA was incubated with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) to add an adenosine at the 3′ terminus, inserted into the pCR4-TOPO vector (Invitrogen), and transformed into chemically competent *Escherichia coli* Stbl two (Invitrogen) according to the manufacturer's instructions. Transformants were screened by PCR for the presence of *Bm*AChE3 cDNA and for orientation of the insert. Plasmid DNAs were purified from bacterial liquid cultures using the QIAprep Spin miniprep kit (QIAGEN, Valencia, CA) and were sequenced using BigDye terminator (Applied Biosystems) with analysis on an ABI3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Clones determined to contain the complete, unaltered coding region served as a source of template for high-fidelity PCR amplification to transfer the cDNA to the pBlueBac4.5/V5-HisTOPO vector (Invitrogen) by using primer pair *Bm*AChE225U19 (5′-CACCATGTACTCGAGGATAGTAG-3′) and *Bm*AChE2067L19 (5′-GGGTTTCAGGTAACTTTTC-3′), according to the manufacturer's instructions. Successful constructs (p*Bm*AChE3) contained the entire coding sequence of *Bm*AChE3 lacking the termination codon and in-frame with the COOH terminus encoding a linker peptide and poly-histidine tail.

Baculovirus Expression. Sf21 insect cells were grown in Graces Insect Medium (GIM, Invitrogen) supplemented with 10% fetal calf serum at 27°C according to instructions provided by the vendor. Sf21 cells, at a density of 1.5×10^6 cells per milliliter, were cotransfected with p*Bm*AChE3 and Bac-N-Blue DNA (Invitrogen) and overlaid with agarose containing GIM and X-Gal or Bluo-Gal according to the manufacturer's instructions. Recombinant baculovirus (blue) plaques were picked and used to infect 5-ml cultures of Sf21 cells that were 25% confluent. For baculovirus-infected cultures expressing r*Bm*AChE3, Sf21 cells were grown in GIM containing fetal calf serum that had been heated for 15–20 min at 65°C to inactivate bovine acetylcholinesterase (GIMΔ). AChE activity was determined 3–5 d after infection by using a microtiter plate assay. The optimum substrate concentration for acetylthiocholine iodide (ASCh; 1.2×10^{-4} M), was determined by titration experiments. The substrate was prepared in 50 mM sodium phosphate buffer, pH 7.5 (phosphate buffer), containing 0.32 mM Ellman's reagent, 5,5′-dithio-*bis*(2-nitrobenzoic acid) (Sigma-Aldrich). The standard assay consisted of 20 μl of enzyme and 180 μl of substrate solution. The reaction was monitored with a Dynatech MR5000 plate reader. GIMΔ was used as a negative control for the acetylcholinesterase assay. Absorbance at 405/630 nm was measured at 0, 5, 10, 15, 30, and 60 min after addition of substrate.

Viral stocks were prepared and titer determined by plaque assay as described in the Bac-N-Blue transfection kit Instruction Manual (Invitrogen). Sf21 cultures were infected with recombinant baculovirus expressing r*Bm*AChE3 at multiplicities of infection (m.o.i.) of 2, 6, and 12 plaque-forming units (PFU) per cell. Samples of the infected cultures were withdrawn at 24-h intervals for 3 d and frozen at −70°C until assayed for AChE activity. Because the highest AChE activity was observed between 24 and 48 h postinfection in cultures of high m.o.i., 50-ml Sf21 shake flask cultures were infected at an m.o.i. of 10 and incubated at 27°C. Thirty hours after infection, culture supernatants were collected by centrifugation, assayed for AChE activity, and used to determine biochemical kinetics of r*Bm*AChE3.

Determination of K_m and V_{max} for r*Bm*AChE3. The general assay for AChE activity was conducted in microplates with ASCh and butyrylthiocholine iodide (BSCh) as substrates according to the method described above. The standard assay consisted of 20 μl of enzyme and 180 μl of substrate solution. The reaction was monitored at 405 nm with a Bio-Tek EL808 ultra microplate reader (Bio-Tek Instruments, Winooski, VT) for 1 h with readings every 10 min.

To determine the K_m and V_{max} values for r*Bm*AChE3, substrate concentrations ranged from 480 to 30 μM (serial dilutions). K_m and V_{max} values were calculated with the aid of SigmaPlot Enzyme Kinetics Module 1.2 (Systat Software, Richmond, CA) on the resultant initial velocities (V_o). ASCh was diluted

from 4.0×10^{-2} to 4.0×10^{-6} M to determine the effect of high ASCh concentrations upon r*Bm*AChE3 activity. Hydrolytic activity of r*Bm*AChE3 was measured as described above in the presence of each ASCh concentration.

Specificity of r*Bm*AChE3 Activity—Inhibition by Eserine Sulfate and BW284C51. Specificity of r*Bm*AChE3 activity was determined with the specific AChE inhibitors eserine sulfate (Holmes and Masters 1967) and 1,5-bis (4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide (BW284c51; Felder et al. 2002). Inhibition reactions were conducted with 2×10^{-3}, 2×10^{-4}, 2×10^{-5}, and 2×10^{-6} M eserine sulfate and 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} M BW284C51. r*Bm*AChE3 extract (20 μl) was mixed with 20 μl of inhibitor in a well of a microtiter plate and allowed to incubate for 10 min at room temperature. Substrate (0.12×10^{-4} M ASCh; 180 μl) was added to the well, and the reaction was monitored as described above.

Determination of the Kinetic Parameters of Inhibition for r*Bm*AChE3 with Paraoxon. The inhibition reaction rate of a nonreversible inhibitor, such as an OP, is described by the bimolecular reaction constant k_i, where $k_i = k_2/K_d$. The rate of phosphorylation of the active-site serine hydroxyl group is represented by the phosphorylation constant k_2, and the affinity of enzyme for OP inhibitor by the dissociation constant K_d (Hart and O'Brien 1973). The method for the calculation of these kinetic parameters of inhibition can be found in Chen et al. (2001) and has been discussed in detail by Pruett (2002). Briefly, the rate of r*Bm*AChE3 inhibition was determined in the presence of 0.12 mM ASCh by using six concentrations of paraoxon (3.5, 3.0, 2.5, 2.0, 1.5, and 1.0×10^{-6} M). The progressive inhibition of r*Bm*AChE3 was monitored over time (12 min; readings at 2-min intervals). The natural log of the percent residual r*Bm*AChE3 activity at each reading for each paraoxon concentration was plotted against time. The apparent rate constant (k), the slope of the line for each paraoxon concentration, was determined by linear regression of the data points. The values for k_i, k_2, and K_d were determined by a double reciprocal plot of the apparent rate constants ($1/k$) against the inhibitor concentrations ($1/[I]$ ($1-\alpha$)). The value of α was calculated as $[S]/(K_m + [S])$.

Results

Construction of *Bm*AChE3 cDNA Expression Clone. First-strand *B. microplus* larval cDNA was amplified by two rounds of high-fidelity PCR (nested PCR). This product was cloned into the pCR4 vector for initial screening to generate a full-length cDNA clone, which was confirmed by sequence analysis to be identical to the GenBank sequence for *Bm*AChE3 (accession no. AY267337). This construct was used as template for transfer of the *Bm*AChE3 cDNA to plasmid, pBlueBac4.5/V5-His-TOPO, to construct the baculovirus transfer plasmid p*Bm*AChE3.

Baculovirus Expression of r*Bm*AChE3. *Cotransfection of Sf21 Insect Cells with pBmAChE3 and Bac-N-Blue DNA.* Cotransfection of Sf21 with p*Bm*AChE3 and Bac-N-Blue DNA yielded blue plaques, which were inclusion body negative. Agarose plugs containing these plaques were picked and used to infect 5-ml Sf21 cultures. The cultures were incubated for 4 to 5 d, and an aliquot of culture medium (including cells) was removed for AChE activity assay, all of which were positive. Two of the AChE-positive cultures were picked for expansion and production of high-titer viral stocks. Baculovirus stock cultures were titered by plaque assay and found to exhibit 2×10^7 PFU/ml.

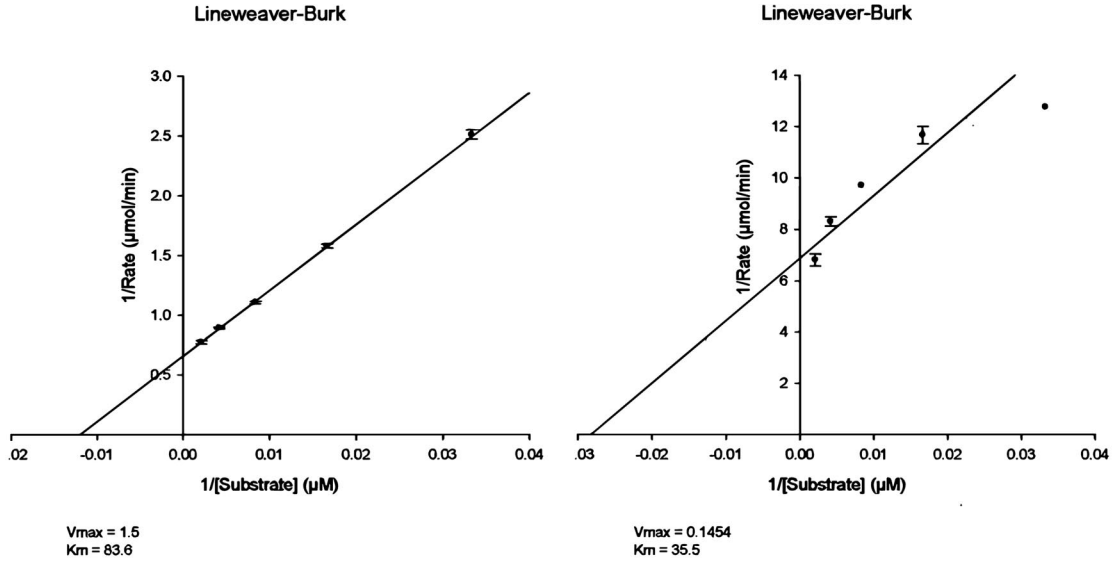

Fig. 1. Calculation of K_m and V_{max} for recombinant *Bm*AChE3 with substrate ASCh (20 μl; $R^2 = 0.99960$) (left) and BSCh (20 μl; $R^2 = 0.992$) (right). Substrate concentrations used in the assays were 480, 240, 120, 60, and 30 μM for both substrates.

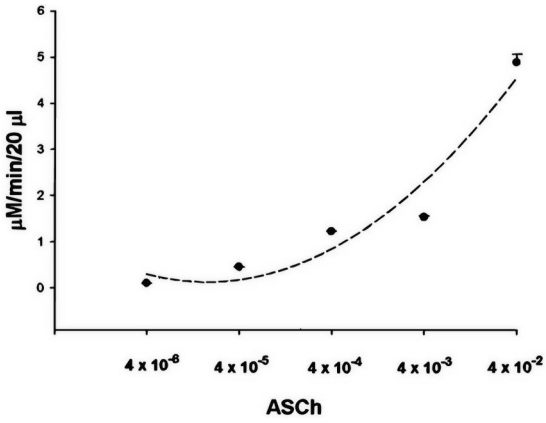

Fig. 2. Activity of r*Bm*AChE3 with increasing concentrations of ASCh.

Expression of rBmAChE3 in Culture Supernatant. Sf21 cultures infected with the recombinant baculovirus were collected and separated by centrifugation into cells and supernatant. Both the cellular and supernatant fractions were found to contain AChE activity (data not shown). Further experiments used culture supernatant as the source of r*Bm*AChE because of its solubility and the relatively higher initial purification compared with the cellular fraction.

Kinetic Analysis of rBmAChE3—Inhibition with AChE-Specific Inhibitors and OP. Kinetic analysis of r*Bm*AChE3 yielded a K_m value of 83.6 μM and a V_{max} of 1.5 μM/min/20 μl with ASCh (Fig. 1). Hydrolysis of BSCh by r*Bm*AChE3 yielded a K_m value of 35.5 μM and a V_{max} of 0.1454 μM/min/20 μl. ASCh ($V_{max}/K_m = 0.0179$) is the "better" substrate, because the V_{max}/K_m ratio is 4.8 times greater than BSCh ($V_{max}/K_m = 0.0041$). The lower K_m value, and a correspondingly lower V_{max} for BSCh indicated a greater affinity for r*Bm*AChE3 than that exhibited for ASCh. High substrate concentrations of ASCh did not inhibit r*Bm*AChE3 activity (Fig. 2).

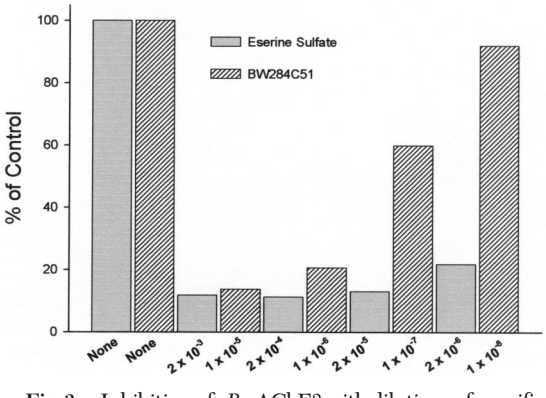

Fig. 3. Inhibition of r*Bm*AChE3 with dilutions of specific inhibitors eserine sulfate and BW284c51 of acetycholinesterase activity.

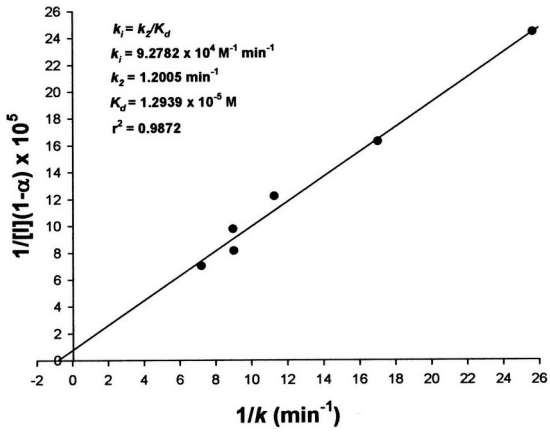

Fig. 4. Double reciprocal plot of rate of inhibition $1/k$ against the inhibitor concentration $1/[I](1 - \alpha)$. The bimolecular rate constant k_i is defined by the equation $k_i = k_2/K_d$ or the slope of the regressed line. K_d is defined by the intercept of the y-axis by the regressed line, and k_2 by the intercept of the x-axis.

The AChE-specific inhibitors eserine sulfate, and BW284c51 inhibited r*Bm*AChE3 (Fig. 3). Eserine sulfate inhibited 88% of AChE activity at 2×10^{-3} M and inhibited 78% of r*Bm*AChE3 activity when diluted to 2×10^{-6} M. BW284c51 inhibited 86% of r*Bm*AChE3 activity at 1×10^{-5} M, but only 8% of r*Bm*AChE3 activity at 1×10^{-8} M.

The calculated bimolecular rate (k_i), phosphorylation (k_2), and dissociation (K_d) constants for inhibition of r*Bm*AChE3 with the OP paraoxon are presented in Fig. 4. There was a strong positive relationship between the independent and dependent variables as reflected by the regression coefficient ($r = 0.9872$).

Discussion

Three putative AChE sequences (*Bm*AChE1, *Bm*AChE2, and *Bm*AChE3) have been previously reported for *B. microplus* (Baxter and Barker 1998, Hernandez et al. 1999, Temeyer et al. 2004); however, these identifications were based on conserved amino acid residues characteristic of AChEs and relatively low amino acid sequence similarity to previously published vertebrate and arthropod AChEs. Indeed, alignment of the amino acid sequences for the three *Bm*AChEs indicates that they did not seem to be closely related to one another, and their identification as genes encoding AChEs lacked certainty (Temeyer et al. 2004). Amino acid sequence information has not been reported in the literature for purified, neural AChE from *B. microplus*, and there have been no mutations reported within the three *Bm*AChEs that correlate with resistance to OPs (Baxter and Barker 1998, Hernandez et al. 1999, Temeyer et al. 2004). However, results of this study indicated evidence for expression of r*Bm*AChE3, the first recombinant ixodid AChE to be expressed.

Detergents are necessary to extract natural AChE from membranes, and in the absence of these detergents, natural AChE is insoluble (Wright and Ahrens 1988). However, the expressed r*Bm*AChE3 in the current study was secreted into culture as a soluble product, presumably because of the incorporation of a carboxy terminus fusion tag that inhibits membrane attachment. Although it is possible that inclusion of a fusion tag to the carboxy terminus of r*Bm*AChE3 may affect its enzymatic activity, similar expression schemes have been used to express AChE in other arthropod species without affecting kinetic properties (Radic et al. 1992, Mutero et al. 1994, Estrada-Mondaca et al. 1998, Villatte et al. 2000).

The AChE identity of r*Bm*AChE3 was validated by biochemical analysis. The AChE-specific ASCh was a more suitable substrate than BSCh for r*Bm*AChE3, indicated by the V_{max}/K_m ratio (Segel 1976), suggesting that r*Bm*AChE3 is an AChE and not a pseudocholinesterase. Unlike vertebrate AChE and other arthropod AChEs (Shafferman et al. 1992), r*Bm*AChE3 was not inhibited by high substrate ASCh concentrations. The r*Bm*AChE3 also was found to be inhibited by the specific AChE inhibitors eserine sulfate and BW284c51 and was very sensitive to paraoxon inhibition. In previous biochemical evaluations of extracted neural AChE from the Muñoz strain of *B. microplus*, successful calculation of the k_i was accomplished with paraoxon concentrations ranging from 3.5 to 1.0 $\times$ 10^{-5} M (Pruett 2002), but these concentrations were much too inhibitory to calculate kinetic parameters of OP inhibition for r*Bm*AChE3 and had to be diluted to 10^{-6} M to allow for successful calculation of the k_i. A plausible explanation for the higher susceptibility of r*Bm*AChE3 to paraoxon inhibition relative to neural AChE extracted from the Muñoz strain may be because of a greater level of general esterases in the tissue extraction that compete with AChE for OP binding. The paraoxon sensitivity of r*Bm*AChE3, which was expressed from a cDNA isolated from the Muñoz strain, is consistent with neural AChE isolated from the same strain. Despite these results, the physiological role of *Bm*AChE3 remains uncertain. We are currently expressing *Bm*AChE1 and *Bm*AChE2 in the baculovirus system to characterize their biochemical properties and identity. Physiological roles of the three *Bm*AChEs may be elucidated through future studies of developmental and tissue-specific expression, gene silencing, and immunohistology.

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